SPERMATOGENESIS IN MARSILIA

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 197

LESTER W. SHARP

(WITH PLATES XXXIII AND XXXIV)

Introduction

In 1912 the writer (6) published the results of an investigation of spermatogenesis in *Equisetum*. The principal conclusion was that the blepharoplasts of bryophytes, pteridophytes, and gymnosperms are derived ontogenetically or phylogenetically from centrosomes. The basis for this conclusion was found in the behavior of true centrosomes and cilia-bearing organs in plants and certain animals, special emphasis being laid upon the remarkable centrosome-like activity of the blepharoplast of *Equisetum*.

The behavior of the blepharoplast of *Marsilia*, as described by SHAW (7) and by BELAJEFF (3) in 1898 and 1899, was also used as a strong argument for the centrosome nature of the blepharoplast. Since these accounts show incompleteness and uncertainty with regard to several points, and since the two writers reached contrary theoretical conclusions, it was deemed advisable to examine certain material[1] at hand with a view toward establishing the true state of affairs in *Marsilia*.

An extensive historical résumé of researches on centrosomes and cilia-bearing structures in plants was presented in the writer's paper on *Equisetum* and will not be repeated here. To the list of papers given should be added those of ALLEN (1) and WALKER (8) on *Polytrichum*. In the present work only those dealing with *Marsilia* need to be reviewed.

The early papers of CAMPBELL (4, 5) on *M. aegyptiaca* and *M. vestita* show the general topography of the male gametophyte. The sequence of wall-formation was not determined in the former species, and great irregularity was reported in the latter. CAMPBELL made out none of the cytological details of spermatogenesis.

[1] The writer is indebted to Dr. C. J. CHAMBERLAIN for a portion of the material used.

In 1898 BELAJEFF (2) described the development of the male prothallium in several Hydropteridineae. In *Marsilia elata* he worked out carefully the exact sequence of walls, which, it will be seen, resembles rather closely that in *M. quadrifolia* as described below. In the latter species BELAJEFF did not figure a developmental series.

SHAW (7) in the same year gave an account of the blepharoplast in *M. vestita*. According to this investigator, a small granule or "blepharoplastoid" appears near each daughter nucleus at the telophase of the second spermatogenous mitosis. During the prophases of the third mitosis it divides and then degenerates in the cytoplasm, while a blepharoplast appears near each spindle pole at metaphase. In the following cell generation (spermatid mother cell) the blepharoplast divides to two which occupy positions near the spindle poles through the fourth or final mitosis. Each spermatid thus receives a blepharoplast. The latter soon shows a small internal granule which multiplies and forms a band; this elongates in close union with the nucleus and bears the cilia. In these facts SHAW found no grounds for the homology of the blepharoplast and the centrosome.

In the following year BELAJEFF (3) reported the results of his researches on *M. macra* and *M. vestita*. He found centrosomes, which he did not hesitate to call them, at the spindle poles in the last three spermatogenous divisions. With regard to the first division he was uncertain; the figure which he gave as possibly representing the first mitosis almost certainly represents the second. He was inclined to identify the centrosome of the second mitosis with the "blepharoplastoid" of SHAW, but believed it to be continuous from the time of its origin, dividing after each mitosis in preparation for the next, and in the spermatid performing the function of a blepharoplast. BELAJEFF regarded this as a strong confirmation of his previously stated theory that the blepharoplast and the centrosome are homologous structures.

In the present paper an attempt will be made to clear up the points left in doubt by these earlier workers, and to add new details which will make it possible to decide between their divergent views concerning the morphological nature of the blepharoplast.

Material and methods

Sporocarps of *Marsilia quadrifolia* were cut open and placed in water at room temperature. Sori were fixed at short intervals until sperms were seen swimming in large numbers, a period of 10 or 12 hours.

Several fixing reagents were employed, including those of Flemming and Benda. The best results were obtained in preparations stained with Haidenhain's iron alum-hematoxylin after the following fixing fluid: 1 per cent chromic acid 25 cc., water 75 cc., glacial acetic acid 1 cc., 2 per cent osmic acid 14 drops.

Description

THE MALE GAMETOPHYTE

A comparison of figs. 1–7 with fig. 8 will do more than a written description to make clear the development of the male gametophyte.

At the time when the gelatinous ring bearing the sori escapes from the sporocarp, the microspore has in section the appearance shown in fig. 1. The nucleus occupies a central position, and large starch grains lie scattered throughout the cytoplasm. These very soon move to the periphery, leaving the nucleus surrounded by a zone of granular cytoplasm. The nucleus then passes to the side of the spore, usually the side opposite the point where the spore met the others of the tetrad, and cuts off a small prothallial cell (wall 1, figs. 2, 3). No "basal cell," such as BELAJEFF (2) figures above the prothallial cell in *M. quadrifolia*, was found in our material.

The next wall (wall 2, fig. 4) passes through the center of the spore. The two hemispherical cells so formed behave alike, each producing an antheridium in the following manner. A curved wall (wall 3, fig. 5) cuts off a large wall cell. A small sterile cell is next formed in the angle between walls 2 and 3 (wall 4, fig. 6). Wall 5 then cuts off a peripheral cell (fig. 7) which completes the wall of the antheridium and defines the limits of the primary spermatogenous cell. There are no centrosomes in any of these mitoses.

Each primary spermatogenous cell by four successive divisions gives rise to a group of 16 spermatids (figs. 9–12). Since the two spermatogenous masses formed in the two hemispheres are entirely

separated from each other by the sterile cells cut off by the walls numbered 4, it seems reasonable to hold with BELAJEFF that the microspore develops two symmetrically placed antheridia separated by wall 2. The prothallial cell degenerates during the later antheridial divisions.

The foregoing account agrees in its essential features with that given by BELAJEFF (2) for *M. elata*, but in that form the development in the two hemispheres is not so symmetrical. In a very few cases we have seen wall 3 in the upper hemisphere strike the spore wall rather than wall 2, which results in a condition more like that in *M. elata*.

SPERMATOGENESIS

As stated above, each primary spermatogenous cell gives rise, by four successive divisions, to 16 spermatids. In these mitoses the chromosomes behave as in ordinary vegetative mitoses, and in view of the purpose of the present study do not require special description. Attention will therefore be directed wholly to the centrosomes and associated structures.

First spermatogenous mitosis.—The primary spermatogenous cell shows nothing which can be called a centrosome. The cytoplasm is dense and contains many small granules, but it is evident that no significant rôle can be attributed to them. The spindle forms without the agency of any visible kinetic center. At late prophase and metaphase it ends rather indefinitely at the poles (fig. 13), but during anaphase these regions appear denser (fig. 14). A little later long and very distinct radiations develop about each pole; at their focus there is a dense and finely granular appearance (fig. 15), but no distinct body is formed. At telophase these polar achromatic structures disappear; in fig. 16 their last remnants may still be seen.

Second spermatogenous mitosis.—In the prophases the second mitosis is similar to the first. The spindle at first ends indefinitely (fig. 17), but during early anaphase it rapidly becomes pointed. Long radiations develop as in the preceding mitosis, and at each pole a very minute and intensely staining granule appears (fig. 18). This is the centrosome. On account of its extremely small size it is practically impossible to make out the exact manner of its origin;

whether it is single from the first or is formed by the union of several granules such as are seen at the poles of the previous mitosis (fig. 15) is a question which must remain in doubt. The cell shown in fig. 18 seems to favor the latter interpretation; at the upper pole there appears to be a small group of granules, while at the lower pole the centrosome is larger and distinctly single.

The centrosomes increase rapidly in size. At very late anaphase they are very conspicuous, and the surrounding radiations form a striking system extending through the greater part of the cell (fig. 19). At telophase the rays become short and faint. The centrosomes are still growing, and in some cases may already show indications of division (fig. 20, upper centrosome).

Third spermatogenous mitosis.—There are now 4 cells in each spermatogenous group, and conspicuous in the cytoplasm of each cell (fig. 21) is the centrosome formed during the anaphase of the preceding mitosis. This centrosome undergoes division at once (figs. 22, 23), in fact this process is often seen beginning during the previous telophase (fig. 20). The two daughter centrosomes rarely diverge from one another. In two or three cells they had moved apart to a distance equal to several times their own diameter, and in one uncertain case they appeared to have reached approximately polar positions. As a rule, however, they degenerate in the cytoplasm without performing any further function. At late prophase and metaphase they can often be made out in the cell (fig. 24), but they bear no relation to the spindle poles, which are at first rather indefinite, as in the first and second mitoses. The persistence of faint radiations about them helps to make their identification sure. During anaphase they usually lie in the cytoplasm at the side of the spindle (fig. 25). The cell shown in fig. 26 contains two pairs of minute granules with very faint rays; since this was the only case of the kind observed, it is impossible to say what it may mean, but it is probable that the two centrosomes which ordinarily degenerate at once have here moved apart and divided again. This renders more certain the interpretation placed upon the paired bodies in figs. 24 and 25. They are doubtless to be identified with the "blepharoplastoid" of SHAW, and are in reality the non-functioning centrosomes.

During anaphase a new centrosome appears at each spindle pole, exactly as in the second mitosis, and behaves in a wholly similar fashion in the subsequent stages. When first discernible it is extremely minute (fig. 25), but very rapidly becomes larger (fig. 26). This formation of new centrosomes after the failure of the old ones is a feature of considerable interest, and will be touched upon again in the discussion.

The division of the centrosome occurs during the telophases, but not always at exactly the same stage, as figs. 27 and 28 show. In fig. 27 the lower centrosome is elongating and the upper one beginning to constrict. In fig. 28 the lower one is still spherical, while the upper one is almost completely divided.

Fourth spermatogenous mitosis.—In the interval between the third and fourth mitoses the centrosomes gradually move apart (figs. 29–31). In view of the rôle which they are to play, they may now be called the blepharoplasts. As they diverge, a delicate central spindle remains between them. The radiations on the side toward the nucleus become stronger, and at the stage shown in fig. 31 form two conspicuous cones of spindle fibers with the blepharoplasts at their apices. The rays extending in other directions are not so well developed as in the previous mitoses.

A marked change now begins in the blepharoplasts. They enlarge, develop one or more internal vacuoles, and become irregular in outline (figs. 31, 32). At late anaphase this process has gone on still farther (fig. 33), and at telophase they may be seen breaking up to several irregular pieces (fig. 34).

Metamorphosis of the spermatid.—The transformation of the spermatid into the spermatozoid seems to take place rather rapidly. The fragmentation of the blepharoplast, begun during the telophase of the last mitosis, continues until a considerable group of pieces has accumulated (fig. 35). These soon take the form of an irregular, lumpy rod, which lies close to the nucleus or against it (fig. 36). A later stage is shown in fig. 37; in this cell, which is noticeably larger than those of figs. 35 and 36, the nucleus has begun to undergo a change in shape which will finally result in the spiral form of the sperm, and the blepharoplast, in close union with it, has formed nearly one complete turn.

As the blepharoplast grows in length it gradually becomes more uniform in thickness without passing through such a regular beaded stage as is seen in *Equisetum*. Our preparations did not permit a more detailed study of this growth, nor was the time of the first appearance of the cilia determined. The blepharoplast often shows a double structure (fig. 37), traces of which are visible in the mature sperm (fig. 42, at middle). SHAW (7) believed this to be an appearance due to the U-shaped cross-section, an idea which is not supported by the present study.

A more advanced stage in the spiral growth of the nucleus and the blepharoplast is shown in side view and in cross-section in figs. 38 and 39. The blepharoplast grows out freely beyond one end of the crescentic nucleus, a feature again clearly brought out in fig. 40, which represents a cell in which the metamorphosis is about half completed. Cilia are easily made out at this stage. As the transformation continues the nucleus and blepharoplast become more closely compacted and are soon very difficult to distinguish.

When the sperm escapes from the spore the coils, about 8 in number, are rather closely wound (fig. 41). The first three or four anterior coils are made up of blepharoplast only and bear no cilia. The remaining coils are composed of both blepharoplast and nucleus and bear cilia upon all but the most posterior regions. The large vesicle, held in the posterior coils, contains the cytoplasm of the spermatid with its inclusions, such as an occasional starch grain and other disorganized material of undetermined nature (figs. 37–41).

As the sperm swims about, it enlarges through the absorption of water, and when it reaches a denser medium, such as the gelatinous material about the megaspore, the coils become more widely separated from each other. Such a sperm, fixed over osmic fumes, is shown in fig. 42. It has a length of 50 μ, while the newly escaped sperm shown in fig. 41 measures but 15.3 μ.

Discussion

The subject of the morphological nature of the blepharoplast was discussed fully in the writer's paper on *Equisetum* (SHARP 6), to which the reader is referred. Two extracts will suffice to make clear the conclusions reached.

Although limited to a single mitosis in the antheridium, the blepharoplast [of *Equisetum*] retains in its activities the most unmistakable evidences of a centrosome nature, and at the same time shows a metamorphosis strikingly like that in the cycads. In thus combining the main characteristics of true centrosomes with the peculiar features of the most advanced blepharoplasts, it reveals in its ontogeny an outline of the phylogeny of the blepharoplast as it is seen developing through bryophytes, pteridophytes, and gymnosperms, from a functional centrosome to a highly differentiated cilia-bearing organ with very few centrosome resemblances [p. 107].

The activities of the blepharoplast in *Equisetum*, taken together with the behavior of recognized true centrosomes in plants and analogous phenomena in animals, are believed to constitute conclusive evidence in favor of the theory that the blepharoplasts of bryophytes, pteridophytes, and gymnosperms are derived ontogenetically or phylogenetically from centrosomes [p. 113].

Let us now turn to the case of *Marsilia*. We have seen that a centrosome first appears at each spindle pole during the anaphase of the second spermatogenous mitosis, and later divides only to degenerate without performing any further function. A new centrosome then appears in the same manner at each pole during the anaphase of the third mitosis, divides, and occupies the spindle poles through the fourth mitosis, and in the spermatid functions as the blepharoplast. This corresponds in the main with SHAW'S (7) description, but that writer failed entirely to see the extensive achromatic structures and the intimate relation they bear to the body in question. Had he done so, it is difficult to understand how he could have failed to recognize the homology of the centrosome and the blepharoplast.

The foregoing features constitute the strongest arguments in favor of the centrosome nature of the blepharoplast. We have as yet found no other case in plants where they are emphasized in so striking a fashion. The body divides at each mitosis and forms the center of an achromatic system unmistakably the same as that accompanying true centrosomes in alga and animal cells. The theory that this is more than mere resemblance and is due to homology surely has a better basis in observed fact and is far simpler than the assumption that a special cilia-bearing organ has taken on characters corresponding in all details to those of centrosomes and has extended itself to three cell generations.

To the present writer it seems clear that we are here dealing with an organ which is gradually passing out of the life history. The farther we pass back from the last spermatogenous mitosis the more indefinite its behavior becomes. The centrosome appearing in the third mitosis is intimately concerned in the formation of the achromatic figure for the fourth mitosis, and before the latter is complete the centrosome has already begun to undergo the peculiar transformation of the blepharoplast. The centrosome appearing at the second mitosis later divides, but only occasionally goes farther. In the first mitosis there seems to be still an abortive start in the formation of a centrosome at anaphase (fig. 15), but no definite body is organized. Even in the earlier mitoses of the male gametophyte a system of rays suggests the presence of a dynamic center at each spindle pole, though no centrosome is present (fig. 4).

These considerations, taken together with other instances in which centrosomes have been reported in several generations of spermatogenous cells (bryophytes), and the large number of cases in which they are limited to the last mitosis (*Equisetum*, *Nephrodium*, cycads), have only served to strengthen our formerly expressed opinion that centrosomes have been partially or wholly eliminated from the early spermatogenous cells, and are retained in so many forms only at the end of the series because of the very important biological function they there perform—the bearing of cilia. They are finally lost altogether when the change from motile to non-motile sperms occurs. Just such a progression as this is seen in passing upward through the bryophytes, pteridophytes, and gymnosperms, and *Marsilia* shows a very instructive stage in the process.

A feature of special interest is the degeneration of the *Marsilia* centrosome just before the third spermatogenous mitosis, and the formation of a new one at each spindle pole. This was also reported by SHAW (7), while BELAJEFF (3) thought there was no break in its continuity after the time of its first appearance. As stated in the description, our material shows at this point some variability, and it is of a sort which tends to reconcile the two earlier accounts. In the majority of cases degeneration begins directly after the

centrosome has divided. In some cases, however, the daughter centrosomes diverge somewhat, and very rarely they may reach polar positions. Although we have observed no metaphase figure of the third mitosis with undoubted centrosomes at the poles, it is nevertheless probable that further search would reveal such cases. It is thus possible that both SHAW and BELAJEFF were correct in their interpretations, that they were dealing with two lots of material showing different behavior at this point.

This is apparently a stage in the life history where the centrosome may be seen in the act of dropping out through failure to carry out its function. A new one forms at each spindle pole for the same reasons that one is developed at the preceding mitosis, where it has been entirely lost from the earlier phases, though as yet it is impossible to determine the nature of these reasons. Since the variable behavior indicates that the organ in question is in all probability a disappearing one, and since there is no organ other than a centrosome which we should expect to see being eliminated from spermatogenous cells, the lack of continuity, if it argues at all, argues for the centrosome nature of the blepharoplast rather than against it.

The conclusions reached as the result of the present investigation are necessarily the same as those stated in the writer's paper of 1912 and cited at the beginning of this discussion. *Marsilia* is even more convincing than *Equisetum* in showing the direct derivation of an advanced cilia-bearing organ from a functional centrosome. Since there is every reason to believe that the blepharoplasts of bryophytes, pteridophytes, and gymnosperms are homologous structures, it follows that they are all "ontogenetically or phylogenetically centrosomes."

Summary

1. In the first spermatogenous mitosis there is present at each spindle pole a dense region with radiations, but no centrosome.

2. During anaphase of the second mitosis a centrosome develops at each spindle pole and at telophase divides to two daughter centrosomes. These only rarely develop farther; they usually degenerate at once in the cytoplasm.

3. In the third mitosis a centrosome develops at each spindle pole at anaphase exactly as in the second mitosis, and during telophase or later divides to two daughter centrosomes.

4. These daughter centrosomes, which may now be called blepharoplasts, move apart and occupy the spindle poles through the fourth or final mitosis.

5. The centrosomes are at all times accompanied by extensive radiations, which in the fourth mitosis give rise to the achromatic figure. When the centrosome divides there is present a central spindle and amphiaster as in animal cells.

6. Before the fourth mitosis is completed the blepharoplast becomes vacuolate and breaks up to a number of fragments. In the spermatid these form a band which elongates spirally in close union with the nucleus and bears the cilia.

7. The evidence afforded by *Marsilia*, together with that gained from other plants and certain animals, is believed to show conclusively that the blepharoplasts of bryophytes, pteridophytes, and gymnosperms are derived ontogenetically or phylogenetically from centrosomes.

The writer is greatly indebted to Professor JOHN M. COULTER for placing at his disposal the facilities of the Hull Botanical Laboratory.

UNIVERSITY OF CHICAGO

LITERATURE CITED

A full list of papers dealing with the subject may be found in the writer's paper on *Equisetum*, cited below.

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EXPLANATION OF PLATES XXXIII AND XXXIV

All figures were drawn at the level of the stage with the aid of an Abbé camera lucida, figs. 1–11 under a Spencer achromatic objective 2 mm. N.A. 1.30 with Zeiss compensating ocular 4, and figs. 13–42 under a Zeiss apochromatic objective 2 mm. N.A. 1.40 with compensating ocular 18. They have been reduced one-half in reproduction, and now show magnifications as follows: figs. 1–11, ×368; figs. 13–42, ×1400.

PLATE XXXIII

FIG. 1.—Microspore at time of liberation from sporocarp: starch grains in cytoplasm.

FIG. 2.—First mitosis in microspore, cutting off prothallial cell (wall 1).

FIG. 3.—Prothallial cell completed.

FIG. 4.—Second mitosis in microspore, forming wall 2 (fig. 8).

FIG. 5.—Third mitosis in microspore, forming walls 3.

FIG. 6.—Fourth mitosis in microspore, forming walls 4.

FIG. 7.—Fifth mitosis in microspore, forming walls 5.

FIG. 8.—Diagram to show sequence of wall formation: the two primary spermatogenous cells marked with nuclei.

FIG. 9.—Two primary spermatogenous cells enlarged.

FIG. 10.—Two-celled stage.

FIG. 11.—Eight-celled stage.

FIG. 12.—Sixteen-celled stage (spermatids).

FIG. 13.—*First spermatogenous mitosis:* late prophase; spindle poles indefinite.

FIG. 14.—Anaphase: cytoplasm denser about poles.

FIG. 15.—Late anaphase: dense regions and radiations present at poles, but no definite centrosomes.

FIG. 16.—Telophase: remnants of polar radiations visible.

FIG. 17.—*Second spermatogenous mitosis:* late prophase; cytoplasm becoming dense and granular at poles of spindle.

FIG. 18.—Anaphase: centrosomes present.

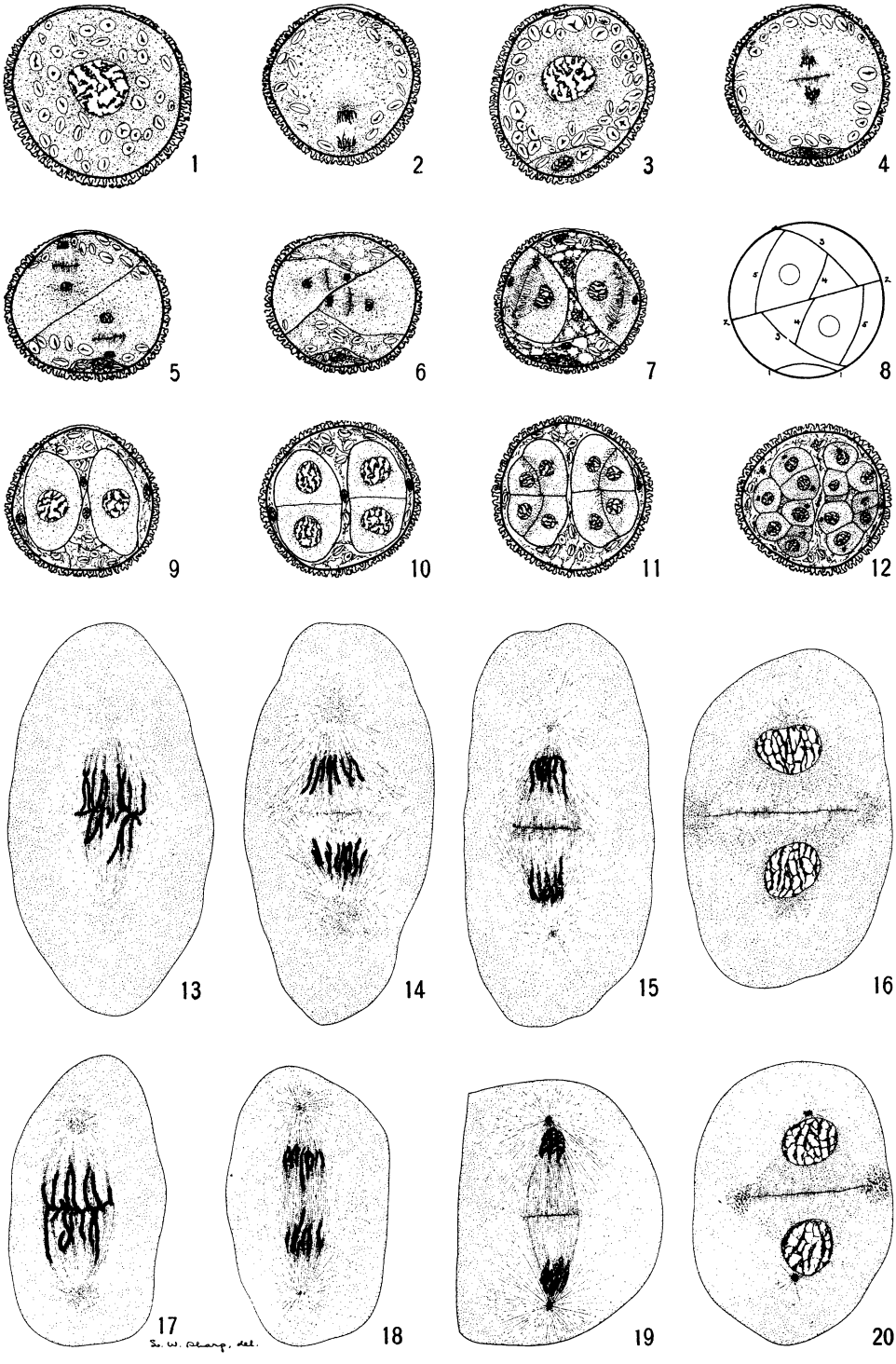
FIG. 19.—Late anaphase: centrosomes and radiations prominent.

FIG. 20.—Telophase: centrosome at upper pole beginning to divide.

PLATE XXXIV

FIG. 21.—Cell of four-celled stage: centrosome undivided.

FIG. 22.—The same: centrosome divided.



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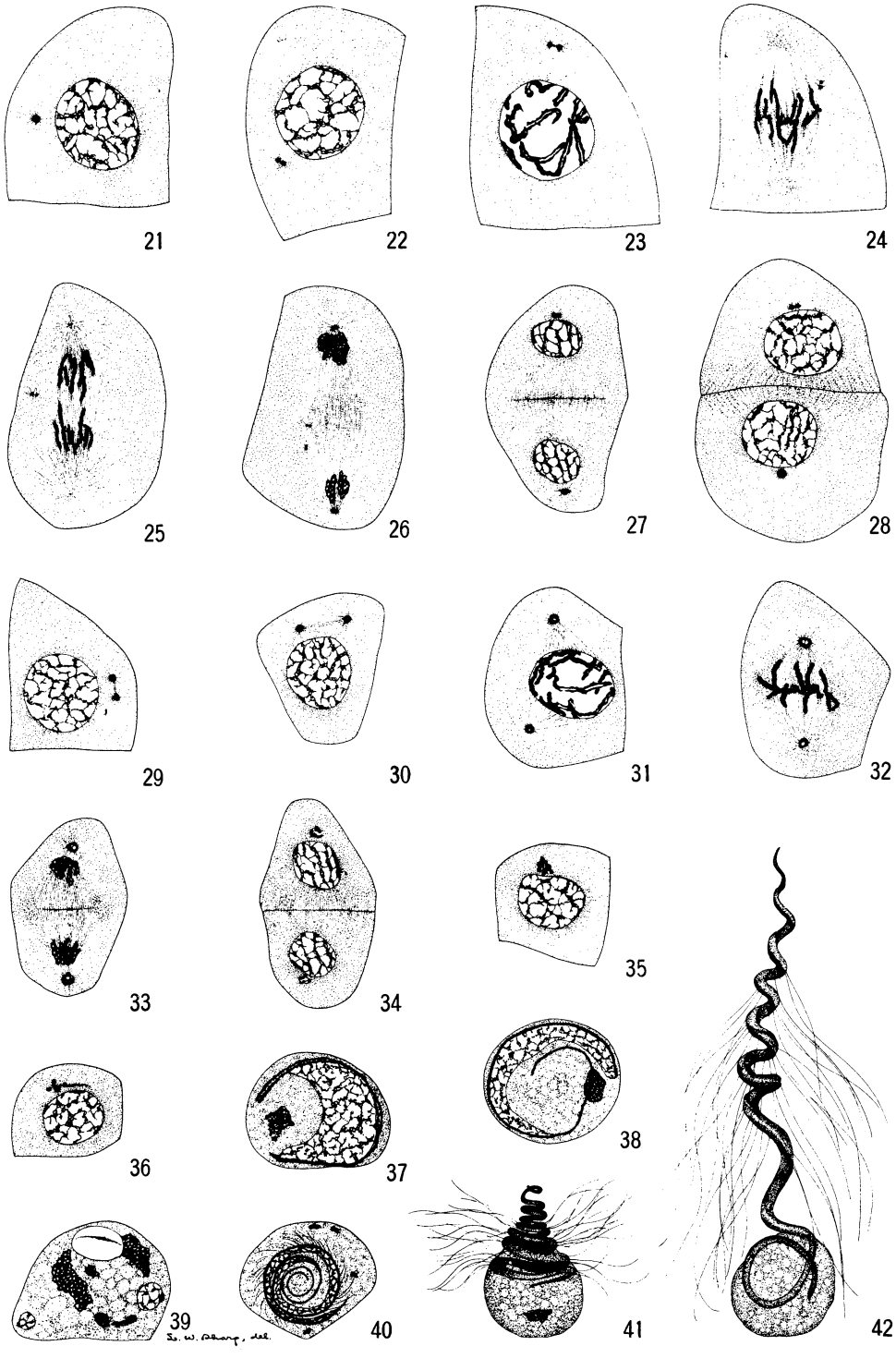

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FIG. 23.—*Third spermatogenous mitosis:* prophase.

FIG. 24.—Late prophase: centrosomes disorganizing in cytoplasm.

FIG. 25.—Anaphase: old centrosomes disorganizing; newly formed centrosomes at poles.

FIG. 26.—"Tassement polaire" stage: old centrosomes have undergone division; new centrosomes greatly enlarged.

FIG. 27.—Telophase: centrosomes beginning to divide.

FIG. 28.—Telophase: division of upper centrosome nearly completed; lower one undivided.

FIG. 29.—Cell of eight-celled stage: centrosomes (blepharoplasts) moving apart.

FIG. 30.—The same, more advanced.

FIG. 31.—*Fourth spermatogenous mitosis:* prophase; spindle forming from radiations; blepharoplast becoming vacuolate.

FIG. 32.—Late prophase: blepharoplast more irregular.

FIG. 33.—Late anaphase.

FIG. 34.—Telophase: blepharoplast breaking up.

FIGS. 35, 36.—Spermatids: blepharoplast has fragmented further and is beginning to form band.

FIG. 37.—Early stage of metamorphosis of spermatid: blepharoplast shows double structure in certain parts.

FIG. 38.—Later stage: blepharoplast grows out freely beyond one end of nucleus.

FIG. 39.—Cross-section of cell in same stage: sections of nucleus with closely appressed blepharoplast at right and left; starch and other disorganized material in cytoplasm.

FIG. 40.—More advanced stage: cilia growing out from blepharoplast.

FIG. 41.—Spermatozoid shortly after escape from microspore: cilia only on middle coils; length 15.3 μ.

FIG. 42.—Spermatozoid in gelatinous material about megaspore, fixed over osmic fumes; length 50 μ.